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Systems for Production of Calves from Cultured Bovine Embryonic Cells

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Abstract. The development of totipotent bovine embryonic cell cultures has great value in cattle breeding. They provide: *(1)* a mechanism for making large numbers of clonal offspring by nuclear transfer; *(2)* an efficient gene transfer system through the use of selectable markers to select transgenic cells; and *(3)* a mechanism for site-specific gene transfer or deletion by homologous DNA sequence recombination. Bovine embryonic cell cultures have been established from blastocyst inner cell mass (ICM) cells, morulae and the precompaction 16–20-cell stage. All have exhibited similar morphology to mouse embryonic stem (ES) cells, pluripotency on differentiation and proliferation in culture. Culture systems have consisted of microdrop loose suspension short-term cultures or long-term cultures on bovine or murine fibroblast feeder layers, in either a microdrop or a culture dish. The relative merit of culture systems or media requirements for mitosis and prevention of differentiation have not been determined. At present, totipotency is also unknown for cultured cells of the 16–20-cell stage. For cultured ICM cells, totipotency was demonstrated by the birth of four calves from ICM cells cultured 27 days or less in a loose suspension microdrop. Advanced pluripotency and perhaps totipotency was demonstrated in one fetus in a recently reported study where morulae cells cultured *in vitro* were chimaerized with non-cultured cells. DNA fingerprinting to associate cell lines with offspring and karyotyping to ascertain chromatin normalcy is important in ES cell research. Data pertaining to the use of each are presented.

Extra keywords: cattle, embryo, cell cultures, pluripotency, totipotency.

Introduction

Embryonic stem (ES) cells have been isolated from the late-stage inner cell mass (ICM) of mouse blastocysts (Evans and Kaufman 1981) or morulae (Eistetter 1989) and cultured on differentiation-inhibiting mouse fibroblast feeder layers to large cell numbers. These ES cells exhibit pluripotency. When chimaerized with normal embryonic cells, they result in ES cell chimaeric offspring and pure ES cell germ line descendants (Evans and Kaufman 1981; Stewart 1991). In mice, ES cells have been used to study embryo development, cell fate and lineage (Joyner 1991). More important to livestock production, ES cells could provide, as they do in mice, a mechanism for gene transfer by transfection, infection or injection of genes into the cells (Evans and Kaufman 1981; Gossler *et al.* 1986; Lovell-Badge *et al.* 1987; Stewart 1991; Anderson 1992). By means of a selectable marker, the transgenic cells could be separated and used by chimaerization into a cleavage stage embryo or blastocyst, as in mice, to produce transgenic offspring (Hooper *et al.* 1987; Joyner 1991; Stewart 1991). Additionally, homologous recombination techniques can be used with cultured ES cells to add or delete genes at specific sites in the genome (Capecchi 1989; Koller *et al.* 1989; Stanton *et al.* 1990; Yagi *et al.* 1990). ES cells also provide a large population of totipotent cells potentially useful for production of clonal offspring by nuclear transfer (First and Prather 1991). This also provides a possible way for production of offspring from transgenic ES cells. Until recently as published in this paper and those of Wheeler (1994) and Sims and First (1993, 1994), evidence of ES cell totipotency and use of ES cells for the above purposes have been achieved only with mouse ES cells (Hooper *et al.* 1987; Capecchi 1989; Koller *et al.* 1989; Stanton *et al.* 1990; Yagi *et al.* 1990; Stewart 1991; Anderson 1992). Cultured ES cells have great value for use in domestic animals to: *(1)* make large numbers of clonal offspring by nuclear transfer; *(2)* provide efficient gene transfer with the use of selectable markers for transgenes; and *(3)* as a mechanism for site-specific gene transfer or deletion through site-specific homologous sequence recombination.

Putative ES cell cultures of cattle, sheep and swine cells have, until recently, demonstrated pluripotency, but not totipotency (Anderson 1992). The exceptions are our recent production of four calves from cultured ICM cells (Sims and First 1993, 1994) and the production of ES cell chimaeric pigs Wheeler (1994).

The development of a totipotent ES cell system depends on: *(1)* identification and isolation of ES cells;

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(2) the ability to inhibit differentiation and commitment to differentiate; *(3)* the ability to culture and passage; *(4)* demonstration of pluripotency on removal of differentiation inhibition; and *(5)* demonstration of totipotency as evidenced by ES cell offspring or offspring from ES cell chimaeras.

This review will focus on some of the variables affecting the isolation, derivation, culture, pluripotency and totipotency of bovine putative ES cells. These variables include: stage of the embryo supplying putative ES cells, methods of ES cell isolation, culture methods and prevention of differentiation, ES cell parentage and descendant identification as well as normalcy of karyotypes.

Establishment of ES Cell Cultures

Most attempts to isolate and culture ICM cells have been based on or adapted from the original methods of Evans and co-workers developed for mice (Evans and Kaufman 1981). These methods have involved placing either the entire Day-4 hatched mouse blastocyst or its ICM after immunosurgical removal of the trophoblast cells onto a murine fibroblast feeder layer treated with mitomycin C and leukaemia-inhibitory factor (LIF). The ICM is allowed to outgrow for 3–4 days and is then picked off and briefly incubated in trypsin to disaggregate the cells.

In bovine, as there is no discrete epiblast some workers have allowed outgrowth for as long as seven days. Conceptually, however, there may be problems associated with this method. First, cells undergoing prolonged association with each other may transcribe information and some may become committed to differentiation before isolation or being influenced by LIF; second, trypsin may be damaging to the cells and their lineage. It is known that mouse ES lines passaged with the use of trypsin can exhibit abnormal karyotypes with frequencies as high as 30–50% (Stewart 1991). These methods also presume that the technician is able to distinguish an ES cell from other cells.

Because these methods have not produced totipotent ES cell lines in domestic animals (Anderson 1992), we chose in the first experiment to physically disassociate the ICM cells by four days after immunosurgery and to culture them as a loose suspension of cells in media (Sims and First 1993, 1994; Tables 1 and 2). The idea is that cell-to-cell contact is required for differentiation and preventing contact should prevent differentiation. There was no apparent differentiation until the ICM cell population growth forced ICM cell contacts in a microdrop at about 1500 cells per microdrop; this resulted in formation of embryoid bodies (Fig. 1). With precompaction morulae, there is no need for immunosurgery or trypsin treatment because the cells are easily and immediately separated by pipetting through a small bore pipette and by micro-needle separation. In our experience, ICM- or morulae-derived cell lines have not usually become established without initially disaggregating the cells.

Table 1. Inner cell mass cells cultured as a loose suspension and used as donors of nuclei in nuclear transfer to produce blastocysts

Adapted from Sims and First 1994

Cell lines	15
Culture duration:	
mean	37 days
maximum	101 days
Nuclear transfers	659
Blastocysts	109 (15%)

Table 2. Inner cell mass cells cultured as a loose suspension and used as donors of nuclei in nuclear transfer to produce blastocysts and calves

Adapted from Sims and First 1994

Cell lines	5
Culture duration	34 days
Nuclear transfers	239
Blastocysts	42 (14%)
After transfer of blastocysts (n = 34) into cows (n = 27):	
No. of cows pregnant at 42 days	13 (49%)
No. of fetuses at 56 day	10 (37%)
No. of calves born	4 (12%)

Culture Methods and Media

Most attempts to culture bovine ES cells have been patterned after the ES cell isolation and culture methods employed for the culture of mouse ES cells and these are essentially the original methods of Evans and Kaufman (1981). ES cells are cultured on mouse embryonic fibroblast cells, usually STO cells, that are mitomycin C-treated to prevent overgrowth of the feeder layer. To this is added murine LIF or STO cells transfected with the gene for murine LIF which promotes mitosis of ES-like cells but prevents their differentiation (Stewart 1991). Most attempts to culture bovine ES cells in this manner have resulted in the demonstration of pluripotency, but not totipotency (Evans *et al.* 1990; Strelchenko *et al.* 1991; Anderson 1992; Saito *et al.* 1992; Stice *et al.* 1994; Strelchenko and Stice 1994) except for the presence of bovine ES cells in a chimaeric fetus (Stice *et al.* 1994). However, the mouse culture system has resulted in extensive proliferation and extensive passage of the cultured cells. It has been suggested that murine LIF and the mouse culture system are not effective in preventing the initiation of differentiation in cultured ES cells of domestic species (Anderson 1992). It is known that the base sequences of DNA of human and murine LIF have 75% homology (Stahl *et al.* 1990). It is also known

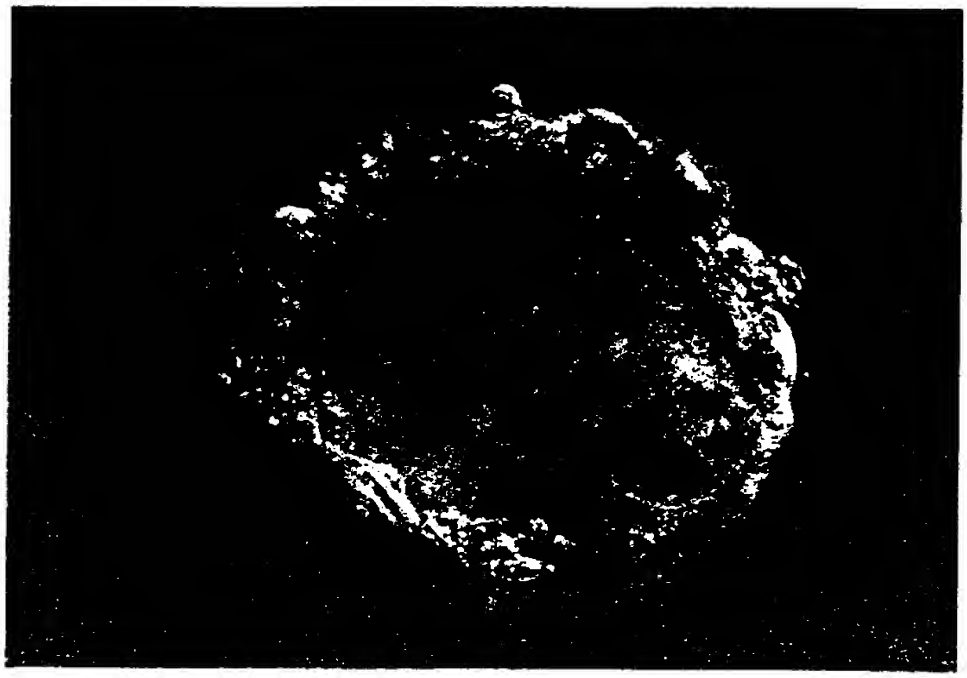

Fig. 1. Bovine embryoid body from microdrop culture of inner cell mass (ICM) cells in loose suspension microdrop culture. With continued culture, these embryoid bodies developed a cystic fluid centre.

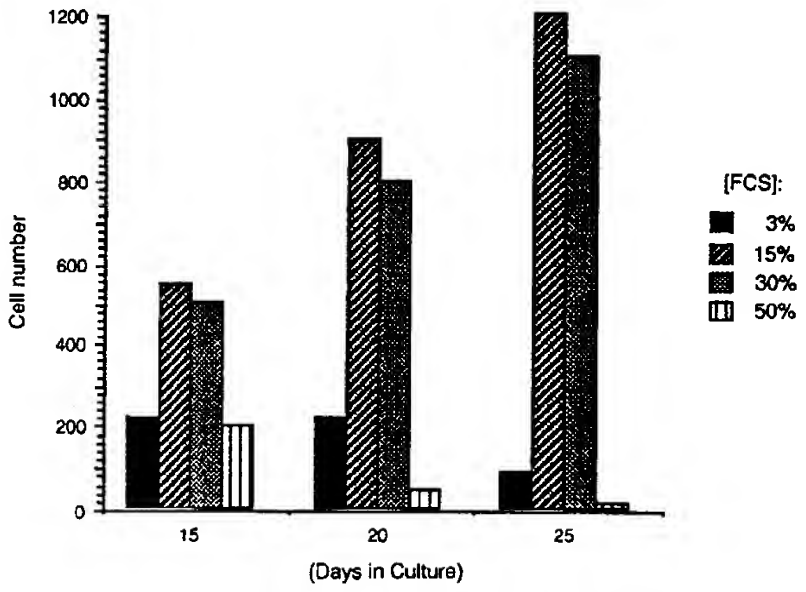

Fig. 2. Effect of concentration of fetal calf serum (FCS) in medium containing CR1aa (Rosenkrans and First 1994) +SIT (selenium–insulin–transferrin) in loose suspension microdrop cultures on proliferation of cultured bovine inner cell mass cells.

that the base sequences of ovine and probably bovine LIF are more like human LIF than mouse LIF (R. L. Williams, personal communication).

It may be that bovine specific culture systems need to be developed. Our first attempt was the use of a loose suspension culture. Mouse ES cells had already been cultured without feeder layers by using fibroblast-conditioned media or cell-free media supplemented with LIF (Pease *et al.* 1990; Stewart 1991). Totipotent embryonal carcinoma lines have also been isolated and cultured in cell-free media without LIF (Mintz and Cronmiller 1981; Stewart and Mintz 1981).

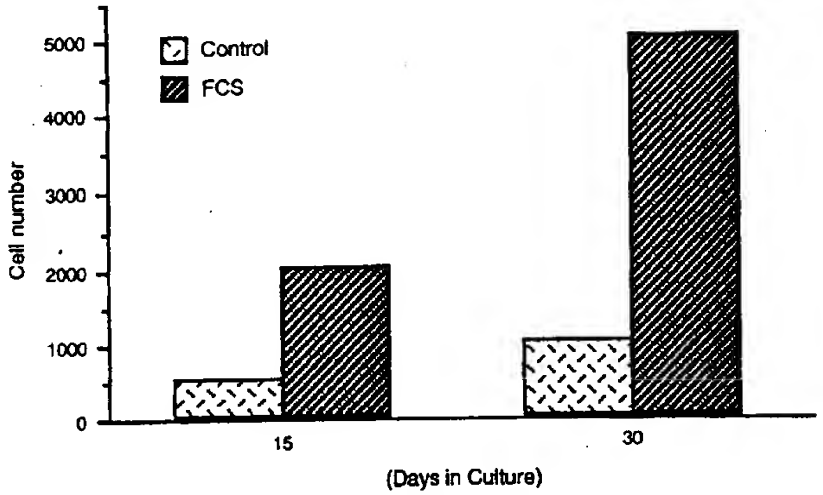

Fig. 3. Population growth of inner cell mass (ICM) cells cultured in loose suspension following the addition of fetal calf serum (FCS) on Day 4 of embryo culture before establishment of ICM cell culture.

Several differentiation-inhibiting and mitotic factors including LIF and buffalo rat liver (BRL) cell-conditioned media were tested in various media combinations for their ability to promote prolonged mitotic activity of ICM cells cultured in loose suspension. Only media consisting of CR1aa (a defined medium of minimal composition including hemisodium lactate and MEM amino acids; Rosenkrans and First 1994) plus SIT (a mixture of selenium, insulin and transferrin) and either glucose, rifampicin and laminin, or 5% fetal calf serum (FCS) supported mitosis through two weeks of culture. Of these, only CR1aa plus SIT plus 5% FCS allowed mitosis and continued proliferation of ICM cells through Week 4. ICM cells from Day 9 and Day 10 bovine blastocysts multiplied *in vitro* when cultured in CR1aa plus SIT and 5% FCS with some lines reaching 2000 cells after two weeks of culture. These cells have the appearance of mouse ES cells—small with large nuclei, little cytoplasm and prominent nucleoli. When removed from non-differentiating conditions and allowed to aggregate, the cultured cells formed embryoid bodies (Fig. 1). These embryoid bodies do not appear to differ morphologically from mouse embryoid bodies.

Although 5% FCS was used to produce the data presented in Tables 1 and 2, the optimum concentration of FCS with this system was later found to be 15% (Fig. 2). An interesting point is that the inclusion of FCS during the last four days of culture for the embryos making up a cell line improved considerably the proliferation of the resultant ICM cells in culture (Fig. 3).

Initial loose suspension culture studies with pooled embryos produced cell lines that proliferated slowly, but were usually no longer viable by five weeks of culture. Live–dead staining at three weeks with calcein AM (live) or ethidium homodimer (dead) showed approximately 80% live cells (green) and 20% dead (red) or dying (orange to yellow) cells whereas at five weeks, nearly 80% were dead or dying. When single embryo lines were cultured in this system, few survived for more than one week (Table 3). Although nuclear transfer blastocysts and offspring were produced from pooled embryo short-term, loose suspension cultures, this method was clearly inadequate for extensive cell proliferation or long-term culture.

Table 3. Effect of number of inner cell masses (cells) used to start a loose suspension culture on inner cell mass cell survival

No. of inner cell masses starting cell line	No. of cell lines Initially	No. of cell lines Surviving at one month
1	159	0
3	241	170 (70·5 %)

We have recently replaced this method with the use of bovine fetal fibroblast cells derived from a 30-day bovine fetus as a feeder layer in a microdrop of the medium containing CR1aa, SIT and 15% FCS previously described. Both ICM and precompaction morulae cells proliferate rapidly and tend to overgrow the feeder layer to form embryoid bodies unless new feeder cells are added at least once a week. Data for establishment of cell lines and early cell proliferation data from this microdrop feeder layer system are shown in Tables 4 and 5. The cultured 16–20-cell stage cells and the feeder layer have both been frozen and reconstituted after freezing. Some cultures have been maintained as long as three months by splitting the cultures and refeeding the feeder layer every ten days. Totipotency of ICM or 16–20-cell stage cells cultured on this feeder layer have not yet been tested.

These culture systems represent a primitive attempt to understanding how to culture bovine ES cells and to understanding the requirements of bovine ES cells for mitosis, differentiation or prevention of differentiation. Much research is yet needed to identify optimum culture conditions for bovine ES cells.

Table 4. Effect of single *v.* multiple 16–20-cell bovine embryos in feeder layer microdrops on establishment of embryonic stem cell lines

No. of embryos per cell line	*n*	No. of cells per drop	No. of cells per embryo	Lines
1	59	17	17	0/59(0%)
3	32	50	16·7	8/32(25%)
5	28	84	16·8	14/28(50%)

Table 5. Effect of number of 16–20-cell precompaction morulae starting a culture on embryonic stem cell survival and proliferation

No. of embryos in cell line	Initially		After 10 days in culture		
	No. of lines	No. of cells	No. of lines	No. of cells	No. of cells per embryo
1	10	21	4	2788	2788
3	16	44	16	25246	8415
5	10	96	10	93334	18667

Stage of Embryos used to Make ES Cell Lines

In mice, successful ES cell lines as evidenced by chimaeric offspring have usually been derived from outgrowth of epiblast cells of the ICM of Day-4 blastocysts as described originally by Evans and Kaufman (1981). There is evidence that late-stage ICMs with higher cell counts more consistently form cultures and totipotent cell lines. Mouse ES cell lines have also been formed from morulae (Eistetter 1989). Totipotent ES cell lines have also been established from murine primordial germ cells and the primordial germ cells of both sexes resulted in chimaeric mice with functional gametes (Stewart *et al.* 1994). We reported recently the birth of four calves from the use of 16–27-day loose suspension cultured bovine ICM cells in nuclear transfer (Sims and First 1993, 1994) (Tables 1 and 2). Identity of the calves was traced to the sires of the respective cell lines by DNA fingerprinting as will be discussed later. This together with the previous evidence that offspring result from use of ICM cells in nuclear transfer in cattle (Keefer *et al.* 1993), sheep (Smith and Wilmut 1989) and rabbits (Collas and Robl 1991) suggests that cultured ICM cells should be totipotent.

Compact morulae are totipotent when used in nuclear transfer (Bondioli *et al.* 1990). However, the totipotent cells are principally inside nonpolarized cells (Navara *et al.* 1991). ES cell lines formed from compact morulae have demonstrated prolonged culture and pluripotency. However, except for one chimaeric fetus so far, their use in nuclear transfer or chimaeras has not resulted in offspring of ES cell parentage (Strelchenko and Stice 1994). Precompaction bovine morulae of the 16–20-cell stage have been used to form cell lines grown on fibroblast feeder layers (Strelchenko and Stice 1994) (Tables 4 and 5). In one report, these lines were derived from single embryos (Strelchenko and Stice 1994). However, in our laboratory, three or more 16-cell embryos have been needed to reliably form a cell line as shown in Tables 4 and 5. These cells should be totipotent because they are derived before commitment for the first differentiation events, cell polarization and compaction (Navara *et al.* 1991). Although their totipotency has not yet been tested, they proliferate rapidly (Table 5) and show evidence of pluripotency when feeder layers are overgrown or removed (Fig. 4).

Effect of Number of Cells on ES Cell Cultures

In mice, the use of more advanced ICM stages with higher cell numbers results in a higher frequency of established cell lines. Our laboratory has compared the efficiency of establishing bovine ICM lines in loose suspension-microdrop cultures from one or three ICMs of hatched blastocysts (Table 3). Seventy percent of the lines started from three embryos were established and survived to one month whereas none of those started from one ICM survived to one month. Others have started cell lines from single ICMs with culture on an STO mouse fibroblast feeder layer (Strelchenko *et al.* 1991; Anderson 1992; Saito *et al.* 1992; Stice *et al.* 1994). The use of a feeder layer in a dish may more efficiently provide for cell growth than loose suspension or feeder layer microdrops.

By means of a bovine fetal fibroblast feeder layer with microdrop culture, we tested the effect of one, three or five embryos from the 16–20-cell stage on the establishment and maintenance of bovine presumptive ES cell lines in two experiments (Tables 4 and 5). In the first (Table 4), 50% of the attempts gave cell lines when started with five embryos, 25% from three embryos and none from a single 16–20-cell embryo. In a second experiment (Table 5), again using one, three or five 16–20-cell bovine embryos and fibroblast feeder layers in microdrops, all of the attempts with cells of three or five embryos per start established cell lines, whereas only 40% of the single embryo starts became established lines.

Across all three experiments, it appears that a critical cell mass of approximately 50–100 cells is needed in microdrop culture to establish ES cell lines reliably. The increase in cell number at ten days by lines from three or five embryos is exponential. The increased proliferation

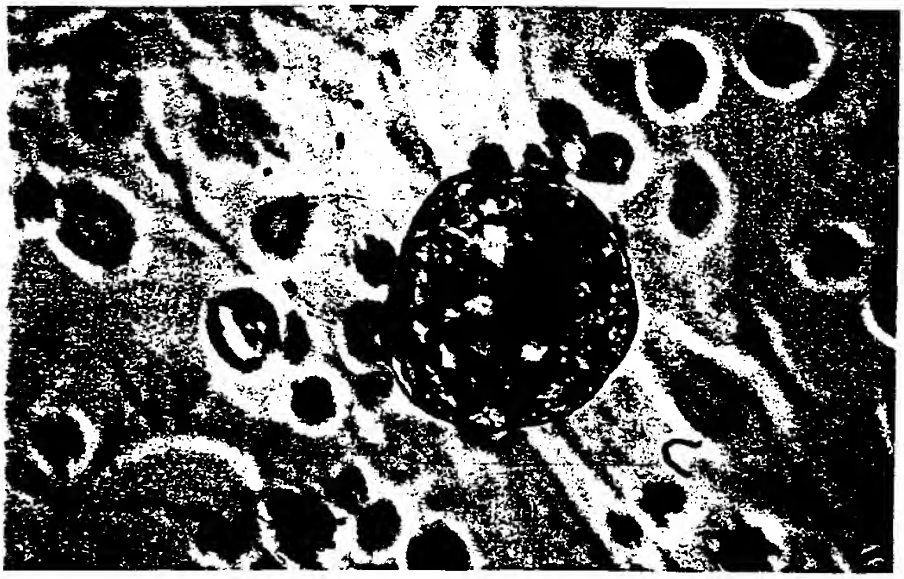

Fig. 4. Bovine embryoid body from microdrop culture of cells from 16–20-cell stage embryo on fibroblast feeder layer.

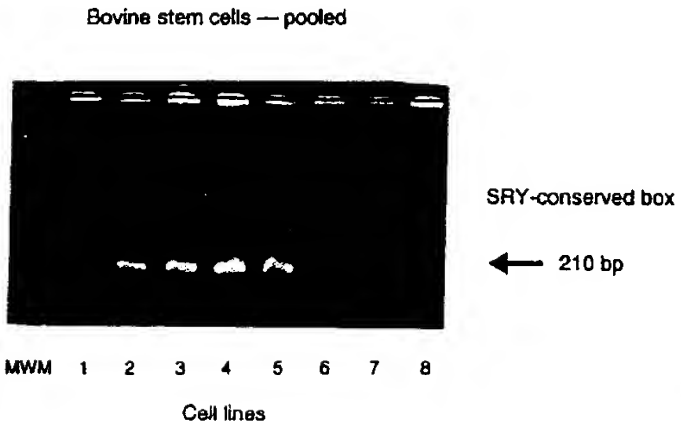

Fig. 5. Cells originating from three embryos or fetal fibroblast feeder layer in a mixed embryo cell line were separately identified by DNA typing. Agarose gel electrophoresis of products of PCR-amplified *SRY* gene (210 bp) from 8 cultures containing stem cells from three pooled bovine embryos each. Results show that each culture had at least one male embryo in its pool. MWM, molecular weight marker.

may be due to synergisms of a critical mass of like cells, synergism of embryos of different genotypes, inhibition of cell cycle when only a few cells are present or the take over of the culture by cells of a dominant embryo. The latter does not seem to be the case. DNA typing of mixed cultures revealed that each mix of cells from three embryos contained cells of the three different genotypes of the embryos mixed to form the cell line and all lines contained at least one male embryo (Fig. 5).

Pooling embryos to start a cell line provides a reliable way of establishing cell lines for research needs such as defining and optimizing culture conditions. Fig. 6 depicts results of DNA typing of cultures consisting of three or five 16–20-cell stage embryos grown on fibroblast feeder layers. Short tandem repeat polymorphism analysis confirmed that descendants of each embryo proliferated and contributed to the culture and each was easily distinguished from the fibroblast feeder layer. However, in commercial use, single embryo lines will be required. This should be possible when the variables affecting ES cell establishment and proliferation are understood.

Genetic and Cytogenetic Identity of Products of Stem Cell Technology

The goal of all stem cell technology is to produce a viable and genetically normal animal. To achieve this

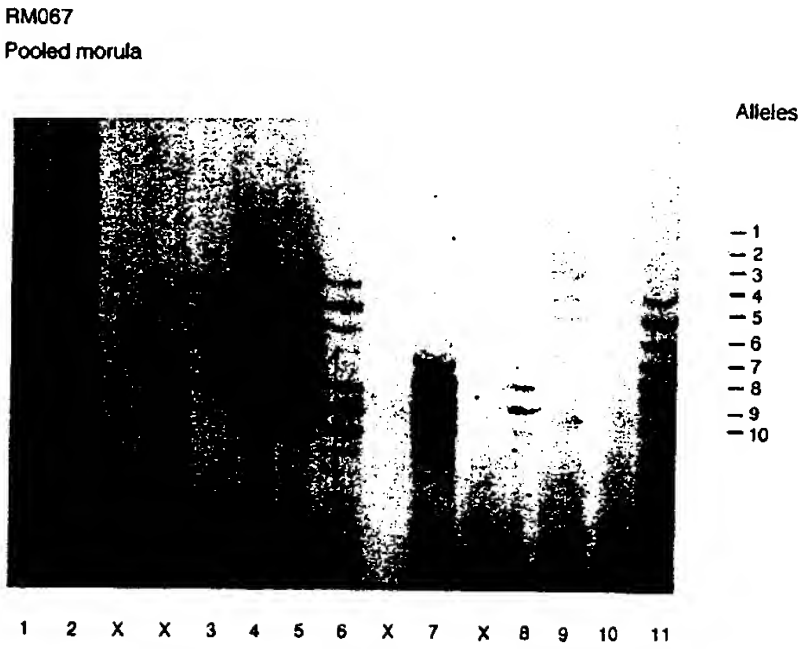

Fig. 6. Denaturing polyacrylamide gel electrophoresis of PCR-amplified silver-stained short tandem repeat polymorphisms detected from RM067 loci. Allelic ladders representing multiple genotypes in cultures containing multiple 16–20-cell stage bovine embryos (1, 2, 3, 4, 5, 6, 7, 8, 9 and 11). Lane 10 is derived from fibroblast feeder layer.

Table 6. Chromosomal abnormalities of karyotyped bovine cells

Calf	Sex	Phenotype	Karyotype at birth	Sire[A]	Dam	1/2 Sibling
1	F	Brangus	60XX/120XXXX[B]	Brahman	Angus hybrid	Calf 2
2	F	Brangus	60XX/120XXXX[B]	Brahman	Angus hybrid	Calf 2
3	M	Longhorn hybrid	60XY	Longhorn	Not known[C]	—
4	F	Holstein	60XX	Holstein	Holstein hybrid	—
Control 1	M	Holstein	60XY	Holstein	Holstein	—
Control 2	F	Holstein	60XX	Holstein	Holstein	—

[A] Karyotype confirmed by short tandem repeat analysis of 11 different loci.
[B] Tetraploidy was <10%.
[C] Not known by karyotype.

goal various laboratories have followed two different pathways beginning with the culture of: *(1)* single or pooled ICMs grown with or without a feeder layer; or *(2)* single or pooled 16–20-cell stage embryos grown with a feeder layer. From the initiation of this pathway of developmental events, it is critical that products of the process are analysed for chromosomal or genetic abnormalities before the continuation of the next sequential stage. Cytogenetic analysis of four neonate bovine calves derived from nuclear transfer of cultured pooled ICM cells grown without a feeder layer revealed the presence of diploid–tetraploid mosaicism in two individuals (Fig. 7 and Table 6). Abnormal ploidy was observed from peripheral blood lymphocytes. Interestingly, by one year of age all calves exhibited normal ploidy. This probably indicates overriding growth of the predominant diploid cell lineage. The calves were normal in gestation length and birth weight. DNA typing of the four calves by means of multiplex short tandem repeats, confirmed their parental lineage (Fig. 8). As noted in Table 6, the four calves were sired by bulls of three different breeds. Thus far, we have no evidence that embryonic cells can be cultured only from a few breeds or sires.

Conclusions

So far, embryonic cell cultures or lines have been established by several methods including loose suspension culture for short-term cultures and, more commonly, murine or bovine fibroblast feeder layers for long-term culture. Pluripotent lines have been derived from 16-cell through blastocyst ICM stages. The efficiency of establishing cell lines and cell proliferation appears to

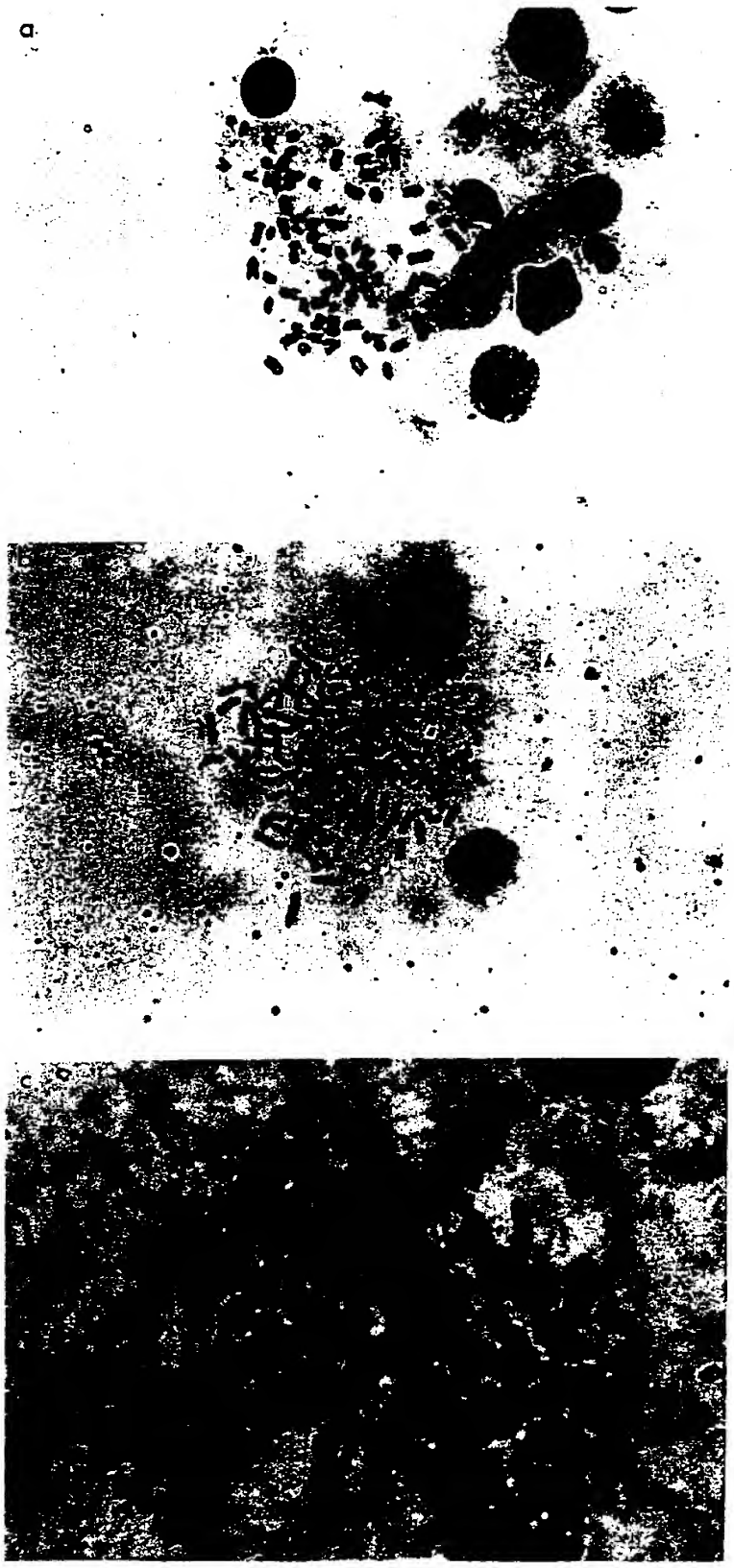

Fig. 7. Diploid–tetraploid mosaicism in two calves derived from cultured bovine inner cell mass cells. *(a)* Normal (2N = 60 XX); *(b)* and *(c)* tetraploid (2N = 120XXXX) (low and high power respectively) spreads from stem cell calves Nos 140 and 141.

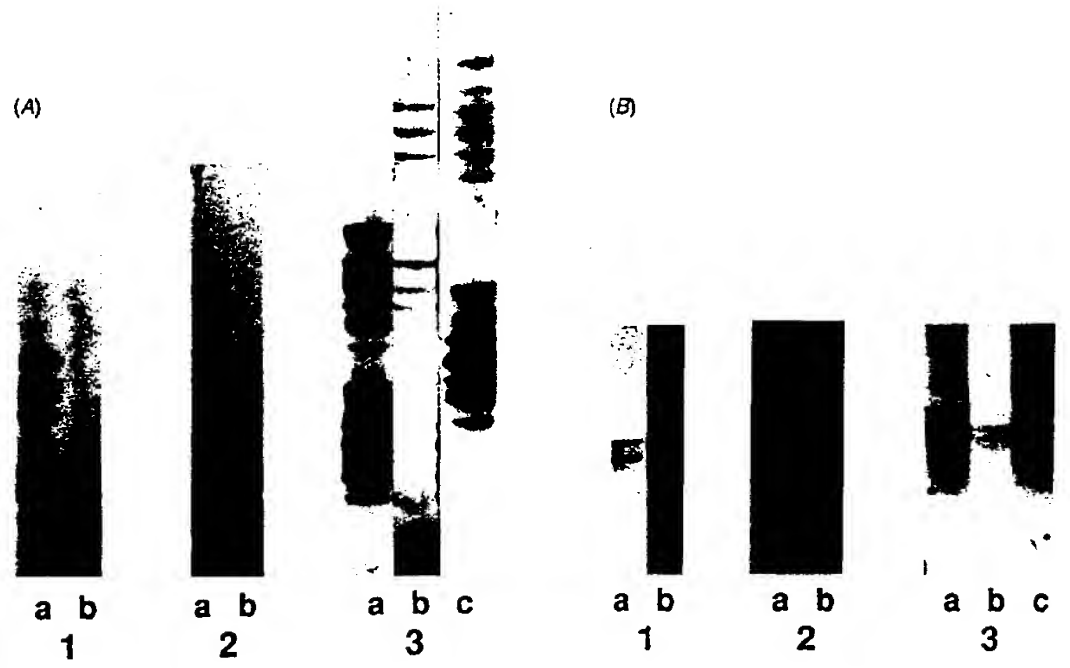

Fig. 8. Stem cell-derived calves. *(A)* Denaturing polyacrylamide gel electrophoresis of PCR-amplified dinucleotide repeat polymorphism RM006 (Kossarek *et al.* 1993). 1a and 1b: Longhorn hybrid male calf No. 144 and sperm from Longhorn bull No. 12199 respectively; 2a and 2b: Holstein female calf No. 136 and sperm from Holstein bull No. 9805 respectively; 3a, 3c and 3b: Brangus female calves Nos 140 and 141 and sperm from Brahman bull No. 9813 respectively. *(B)* Denaturing polyacrylamide gel electrophoresis of PCR-amplified dinucleotide repeat polymorphism D21S4 (Steffen *et al.* 1993). 1a and 1b: sperm from Longhorn bull No. 12199 and Longhorn hybrid male calf No. 144 respectively; 2a and 2b: Holstein female calf No. 136 and sperm from Holstein bull No. 9805 respectively. 3a, 3c and 3b: Brangus calves Nos 140 and 141 and sperm from Brahman bull No. 9813 respectively.

be affected by the number of cells or embryos starting the line. DNA typing has shown that all embryos of a mixed embryo culture contributed to the proliferated culture and embryonic stem cells can be separated by DNA fingerprinting from the feeder cells.

Short-term loose suspension ICM cell cultures produced calves, each with the DNA fingerprint of each sire of the respective calves. DNA-based methods for associating offspring with cell lines or parents of cell lines and karyotypes of cells, fetuses or offspring are both necessary. In some cell lines, fetuses and offspring have exhibited abnormal ploidy. To date, most attempts to produce offspring from ICM- or morulae-derived ES cells cultured with long-term STO cell feeder layers, have resulted in pregnancy failure in the first trimester when ES cells were used in nuclear transfer or have failed to retain ES cells in the progeny produced by chimaerization. The exception is one chimaeric fetus derived from the use of morula ES cells in chimaerization with early embryonic cells.

There is a great deal yet to learn about ES cell culture requirements for maintenance of totipotency. If bovine ES cell lines lose imprinting pattern and totipotency with long-term culture and passage as suggested for mouse ES cells by Pedersen (1994), we may be limited to the use of short-term cultures for multiplication of embryos and efficient production of transgenic animals. To date, no bovine ES cell system has met all of the criteria indicated for a totipotent ES cell line.

Acknowledgments

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